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CELL BIOLOGY – IMMUNOLOGY – PATHOLOGY

Proteinase 3 sidesteps caspases and cleaves p21$^{Waf1/Cip1/Sdi1}$ to induce endothelial cell apoptosis

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Background. Emerging data raise possibilities of a complex and specific biologic role for leukocyte-derived proteases in substrate processing and in signaling pathways. Neutrophil proteinase 3 (PR3) is a caspase-like protease that enters endothelial cells, cleaves nuclear factor-κB (NF-κB), and induces sustained JNK activation, implying that the major cell cycle inhibitor p21 may be inactivated. Cleavage of p21 by caspase-3 is reported to be required for endothelial cell apoptosis. We hypothesized that PR3 may target p21.

Methods. Human umbilical vein endothelial cells (HUVEC) were treated with or without PR3 (5 μg/mL) from 0 hours or up to 8 hours, and analyzed for changes in cell cycle control proteins by immunoblotting, immunofluorescence and flow cytometry.

Results. PR3 exposure resulted in cleavage of p21 between Thr^{80} and Gly^{81}, loss of nuclear p21 by cytoplasmic sequestration and depletion of p21 from cyclin/cyclin-dependent kinase (CDK) complexes. Examination of cyclins D and E, p53, Rb, and p27 revealed a largely nonproliferative expression profile. Cells arrested in G_1 were more susceptible to PR3 effects. We examined inflamed human colonic tissue and found a fragment similar in size to that generated by PR3 in HUVEC. Granzyme B, a T-cell homologue of PR3 that cleaves caspase substrates, also cleaves p21 between Asp^{62} and Phe^{63}. A reported substrate of granzyme B and caspases, Bid, is cleaved by PR3 signifying commonality of substrates among these proteases.

Conclusion. A theme is developing that the granulocyte protease, PR3, is an exogenous caspase-like molecule that can sidestep intracellular caspase functions at sites of inflammation.

The inflammatory response to tissue injury, due to either trauma or infection, is primarily characterized by leukocyte recruitment and extravasation. Although these cells are critical to resolution of the injury, they are often responsible for the perpetuation of inflammation. A specific subset of leukocytes, namely neutrophils and monocytes, possess granules containing millimolar amounts of a variety of proteolytic enzymes that can be harmful to tissues when released extracellularly [1]. Although protease inhibitors exist in plasma and in tissue fluid to offer some defense, protease concentrations can overwhelm the inhibitors and exist as unbound, active proteases at high concentrations [2, 3].

Until recently, it was thought that the mechanism responsible for vascular endothelial cell injury was solely due to the proteolytic degradation of extracellular matrix molecules by these enzymes [4]. It is now becoming clear that the mechanisms underlying tissue injury are more complex than initially thought. We became interested in proteinase 3 (PR3) with the discovery that a subset of vascular diseases is associated with anti-PR3 autoantibodies, termed antineutrophil cytoplasmic autoantibodies (ANCA) [5, 6]. ANCA are hypothesized to bind to PR3 expressed on the surface of neutrophils and monocytes, causing an exacerbation of the degranulation process, resulting in excessive release of proteases, thus contributing to endothelial injury and vasculitis [7, 8]. PR3 is a granule serine protease that specifically processes multiple biologic substrates, including angiotensinogen [9], transforming growth factor-β1 (TGF-β1) [10], tumor necrosis factor-α (TNF-α) [11, 12], C1 inhibitor [13], and the transcription factor Sp1 [14].

PR3 can traverse the endothelial plasma membrane, and, once internalized, induce apoptosis, implicating that PR3 has diverse roles yet to be identified [15]. Currently what is known about PR3-mediated endothelial cell apoptosis is that both the catalytically inactive and active forms of PR3 induce death; however, the kinetics of death are quite different. Inactive PR3 causes endothelial cell apoptosis within 12 to 24 hours [15]. Although little is known as to the specifics of this event, we found that a 100 amino acid region of the PR3 molecule is implicated as responsible for death since a C-terminal fragment of

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PR3 induces apoptosis in a time frame similar to that of the full-length inactive molecule.

Exposure of endothelial cells to active PR3 results in cleavage and inactivation of the transcription factor, nuclear factor-κB (NF-κB), and sustained activation of JNK [16]. Inhibition of caspases did not block the cleavage of p65 NF-κB, and sequence analysis showed that the PR3 cleavage site was unique with respect to reported caspase cleavage sites [17]. Death as a result of active PR3 treatment generally results in 4 to 6 hours.

These findings led us to hypothesize that neutrophils and monocytes carry proteases that can function like caspases. In support of this hypothesis are reports that PR3 can mimic caspase-2 and -3 by cleaving the Sp1 transcription factor [18], and mimics caspase-1 in its ability to process interleukin-1β (IL-1β) [12]. Such a function would provide a unique mechanism of cross talk between leukocytes and endothelial cells at sites of inflammation that would impact both cytokine networks and cell viability.

While cleavage of NF-κB is required for apoptosis, cleavage alone is not sufficient [16, 19]. These observations suggest that PR3-induced apoptosis may involve cleavage of other, yet unidentified, substrates. A logical candidate is $p21^{Waf1/Cip1/Sdi1}$ (p21). p21 appears to be a major determinant of cell fate [20]. It is known to inhibit the activation of the apoptosis signal-regulating kinase, c-Jun N-terminal kinase 1 (JNK 1), and caspase-3, and it exerts an antagonistic effect on the mitochondrial pathway of apoptosis [20–23]. In line with these data, we reported earlier that exposure of endothelial cells to PR3 results in sustained activation of JNK [16], and for this to occur, p21 inactivation would be implied, thus leading to the proposal that PR3 may target p21. Furthermore, the need for inactivation of p21 by caspase-3 cleavage is reported to be required for endothelial cell apoptosis [17].

The studies here determine if PR3-mediated cleavage of p21 is a component of PR3-induced human umbilical vein endothelial cell (HUVEC) apoptosis. Because p21 is an inhibitor of cyclin/cyclin-dependent kinase (CDK) complexes responsible for cell cycle progression, we determined what impact p21 cleavage could have on cell cycle regulation in the presence of PR3. As an extension, we investigated whether the T-cell protease granzyme B, highly homologous in sequence and in structure to PR3, had related substrate specificities. We also addressed the issue of whether this phenomenon is relevant to sites of inflammation in vivo by examining tissue for the presence of p21 cleavage products.

METHODS

Antibodies

Antibodies used were rabbit anti-Bid (FL-195), mouse anti-cyclin D1 (HD11), mouse anti-cyclin E (HE12), goat anti-ERK 1 (K-23), mouse anti-p21 (187), rabbit anti-p21 (C-19), rabbit anti-p27 (N-20), rabbit anti-p27 (C-19), mouse anti-p53 (DO-1), rabbit anti-Rb (C-15) (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), rabbit anti-WAF1 (Ab-5) (Calbiochem, La Jolla, CA, USA), mouse anti-pRb (Pharmingen, San Diego, CA, USA) mouse anti-Cip1/WAF-1 (Upstate Biotechnology, Waltham, MA, USA) and rabbit anti-PR3 serum (kindly donated by Wieslab AB, Lund, Sweden).

Culture and treatment of HUVEC

Pooled HUVEC (Clonetics, San Diego, CA, USA) were cultured in endothelial basal medium (EBM) plus endothelial growth medium (EGM) BulletKit supplements (Clonetics) for four to eight doublings, but no longer than 2 to 3 weeks. Triton X-100 was removed from proteolytically active PR3 using an Extracti-Gel D AffinityPak detergent-removing column (Pierce Chemical Co., Rockford, IL, USA). HUVEC monolayers were washed with phosphate-buffered saline (PBS) and treated with 5 μg/mL of PR3 (Wieslab AB) at 37°C. Of note, a second source of commercial native PR3 purified from purulent sputum was as efficient as purified neutrophil-derived PR3 from whole blood (Elastin Products Co. Owensville, MO, USA).

Because serum contains inhibitors of serine proteases, all treatments were performed in serum-free EBM medium without supplements. For competitive broad-spectrum inhibition of caspases, YVAD-*fmk* (Calbiochem) was added at 100 nmol/L for 2 hours prior to and during protease treatments as indicated. Cells were treated with bleomycin (Sigma Chemical Co., St. Louis, MO, USA) prepared as described [24] at 1 μg/mL in standard growth medium 24 hours prior to protease treatments.

Assessment of 5-bromo-2′-deoxyuridine (BrdU) incorporation and caspase-3 activation by flow cytometry

BrdU was added to cell monolayers at 10 μmol/L for 30 minutes prior to harvest. Attached and detached cells were collected and combined for analysis. Apoptosis was quantitated as previously described [16]. Briefly, detached and attached cells, treated for 8 hours with 5 μg/mL PR3 in serum-free medium, were combined, fixed, and stained using the Fix and Perm Kit as directed (Caltag, Burlingame, CA, USA). Apoptotic cells were tagged using α-activated caspase-3 antibody plus fluorescein isothiocyanate (FITC)-conjugated goat α-rabbit secondary antibody (Dako, Carpinteria, CA, USA). All labeled cells were analyzed by FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) linked to a Cicero/Cyclops system (Cytomation, Fort Collins, CO, USA).

Immunoblot analysis and immunocytochemistry of HUVEC

Immunoblotting was performed as previously described [16]. Briefly, diisopropylfluorophosphate (DFP) was added to the treated HUVEC cultures 5 minutes before cell lysis to irreversibly inhibit PR3 activity. Cells were lysed at 3×10^6 cells/mL in hot Laemmli protein lysis buffer [25], boiled (5 minutes), and sonicated. Two $\times$ 10^5 cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Primary antibodies were prepared in 1% Blotto and incubated overnight at 4°C. Horseradish peroxidase-conjugated secondary anti-mouse, anti-rabbit, or anti-goat antibodies (Chemicon, Temecula, CA, USA) were incubated for 2 hours at room temperature, and visualized using West Pico chemiluminescence substrate (Pierce Chemical Co.). For immunocytochemistry, HUVEC were plated on 20 mm cover slips at a density of 7.5×10^4 cells/slip and grown for 48 hours. Following treatments, cover slips were prepared and visualized as described [15] using anti-p21 antibodies.

In vitro cleavage of p21, $p27^{Kip1}$, and Bid

Recombinant hexahistidine-tagged human p21 was metallopurified as described previously [26], immobilized on 50 μL of Talon resin (Pierce Chemical Co.), washed three times with PBS and incubated with either 3 μg of PR3 or 10 U Granzyme B (Biomol Research Laboratories, Plymouth Meeting, PA, USA) for 30 minutes at 37°C. Identical conditions were used for Bid, which was immunopurified from HUVEC lysate (1×10^7 cells prepared by lysis in RIPA buffer) using the anti-Bid antibody conjugated to protein A/G beads (Pierce Chemical Co.). Reactions were terminated with hot 2× Laemmli sample buffer and boiled for 5 minutes. Samples were subjected to SDS-PAGE and visualized by Coomassie stain or further evaluated by immunoblotting using N-terminus-specific anti-p21 antibody. Fragments of p21 were sequenced from polyvinylidine difluoride (PVDF) membranes by Edman degradation by the Microprotein Core Facility at the University of North Carolina at Chapel Hill.

Immunoprecipitations

For each sample, 1×10^6 HUVEC were scraped in 750 μL immunoprecipitation (IP) buffer [25 mmol/L HEPES pH 7.5, 12.5 mmol/L $MgCl_2$, 150 mmol/L KCl, 0.5% Igepal, 10 mmol/L dithiothreitol (DTT), 10% glycerol and protease inhibitors], passed three times through a 25 gauge syringe, nutated for 1 hour, and insoluble debris removed by centrifugation at 5000 $\times$ *g* for 10 minutes at 4°C. Two micrograms antibody for p21, cyclin D, or an isotype-matched nonspecific control antibody was added and incubated overnight at 4°C. Fifty microliters protein A/G slurry (Pierce Chemical Co.), washed three times in IP buffer, was added to sample, incubated for 1 hour, pelleted by centrifugation at 3000 $\times$ *g* for 3 minutes at 4°C, washed three times in IP buffer, and bound proteins and antibodies were removed from beads with 1.5× Laemmli sample buffer, boiled for 5 minutes, and subjected to SDS-PAGE and immunoblotting.

Human tissue preparation for immunoblotting

Small-sized pieces of affected colon tissue and tissue distal to the inflamed site ($\sim$1.0 cm^3) were removed from Crohn's disease and ulcerative colitis patients during operation and immediately frozen in liquid nitrogen. Samples were homogenized in PBS or IP buffer containing ethylenediaminetetraacetic acid (EDTA)-free complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), and subjected to either immunoblotting or IP, respectively. Immunoblotting was performed for PR3, p21, and truncated p21.

RESULTS

PR3 cleaves p21 after internalization

To determine if p21 is a substrate of internalized PR3, endothelial cells exposed to PR3 were analyzed for p21 cleavage products. The serine protease inhibitor DFP was added 5 minutes prior to cell lysis to prevent spurious cleavage of proteins, as previously described [16]. Results revealed a distinct cleavage product of p21 by 2 hours that persisted through 8 hours, which was not present in mock-treated control cells (Fig. 1A). The p21 cleavage product, as detected by an N-terminus-specific antibody, was approximately 10 kD. An increase in apoptosis over mock-treated controls was observed (Fig. 1B) coincident with p21 cleavage, and this death was consistent with our previous findings [15]. To map the cleavage site, recombinant human p21 was incubated with PR3 in vitro and resultant peptide fragments were extracted and sequenced (Fig. 1C). Sequence analysis revealed a unique cleavage site on the p21 molecule between amino acids Thr^{80} and Gly^{81}. The identified site was unique from that reported for caspase-3, which cleaves between amino acids Asp^{111} and Leu^{112} (Fig. 1D). From this set of experiments, we conclude that PR3-mediated endothelial cell death involves cleavage of p21 during activation of apoptosis.

PR3 cleavage of p21 does not require caspase activity

PR3 cleaves p21 in vitro; however, the possibility remains that in vivo PR3 could activate some other caspase that then cleaves p21 at or around this same site. Therefore, we examined whether intracellular p21 cleavage could occur in the presence of the broad-spectrum caspase inhibitor, YVAD-*fmk*, to determine if caspases contribute to p21 cleavage events associated with

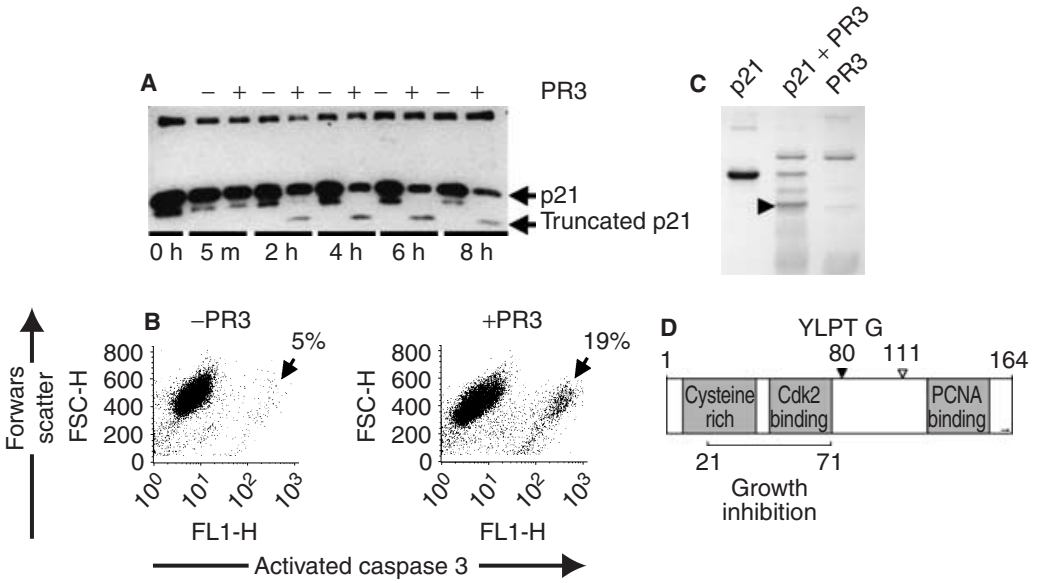

Fig. 1. Proteinase 3 (PR3) cleaves p21 in endothelial cells. (*A*) Immunoblot analysis using an amino terminus-specific anti-p21 antibody reveals a ~10 kD p21 cleavage product in PR3-treated cells. (*B*) PR3-induced apoptosis is coincident with cleavage as determined by flow cytometric analysis of apoptotic cells. (*C*) In vitro cleavage of p21. Recombinant human p21 was incubated with human PR3 and resultant peptide fragments visualized by Coomassie stain of an 18% sodium dodecyl sulfate (SDS) gel. Cleavage product was extracted and sequenced (arrowhead). (*D*) Schematic representation of p21 shows functional domains, the PR3 cleavage site (closed arrowhead) and the reported caspase-3 cleavage site (open arrowhead).

PR3 exposure. We first confirmed that YVAD-*fmk* was effective at inhibiting caspases in HUVEC (Fig. 2A). No activated caspase-3–positive cells were detected in the presence of the inhibitor. Immunoblot analysis revealed that PR3 cleaved p21 in the presence or absence of YVAD-*fmk* (Fig. 2B). These data confirm that PR3-induced p21 cleavage is caspase-independent and supports indications that p21 is a substrate of PR3.

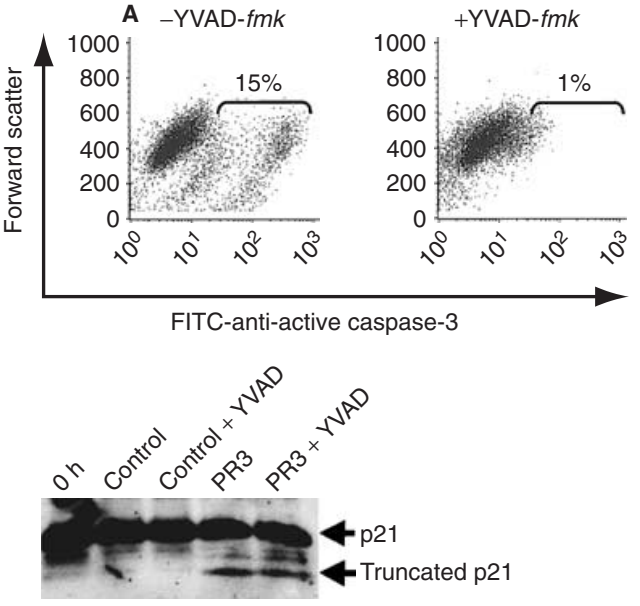

Fig. 2. Proteinase 3 (PR3)-mediated cleavage of p21 is caspase independent in vivo. (*A*) YVAD-*fmk*, a pancaspase inhibitor, efficiently inhibits caspases in human umbilical vein endothelial cells (HUVEC) as determined by flow cytometric analysis of cells positive for active caspase-3. (*B*) Cleavage of p21 in PR3-treated HUVEC is observed at 4 hours in cells pretreated for 2 hours with or without YVAD-*fmk*.

PR3-treated endothelial cells reside primarily in G_0/G_1 stage of the cell cycle

We observed that shortly after PR3 exposure cells began to round up taking on a morphologic appearance of mitotic cells with doublet-like cell clusters. We considered that this might be due to decreased attachment since PR3 can cleave extracellular matrix proteins; however, we repeatedly observed that cells were not apoptotic at this point (data not shown). This change in morphology could be indicative of PR3-induced cell cycle perturbation; therefore, we determined if p21 cleavage was causing premature entry into S phase. We measured the percentage of BrdU-incorporating S-phase cells by flow cytometry and found that no increase in BrdU incorporation was observed as compared to mock-treated controls (data not shown). These results would be in agreement with what was observed after caspase-mediated p21 cleavage [17]. To further characterize changes in cell cycle status after PR3 exposure, we investigated cell cycle regulating proteins (Fig. 3A), including cyclin D and cyclin E. Cyclin D normally increases during G_0/G_1 reaching maximal levels in S phase, and then remains elevated until mitosis. Cyclin E normally starts to accumulate late in G_0/G_1, peaks at the G_1/S transition and then decreases during

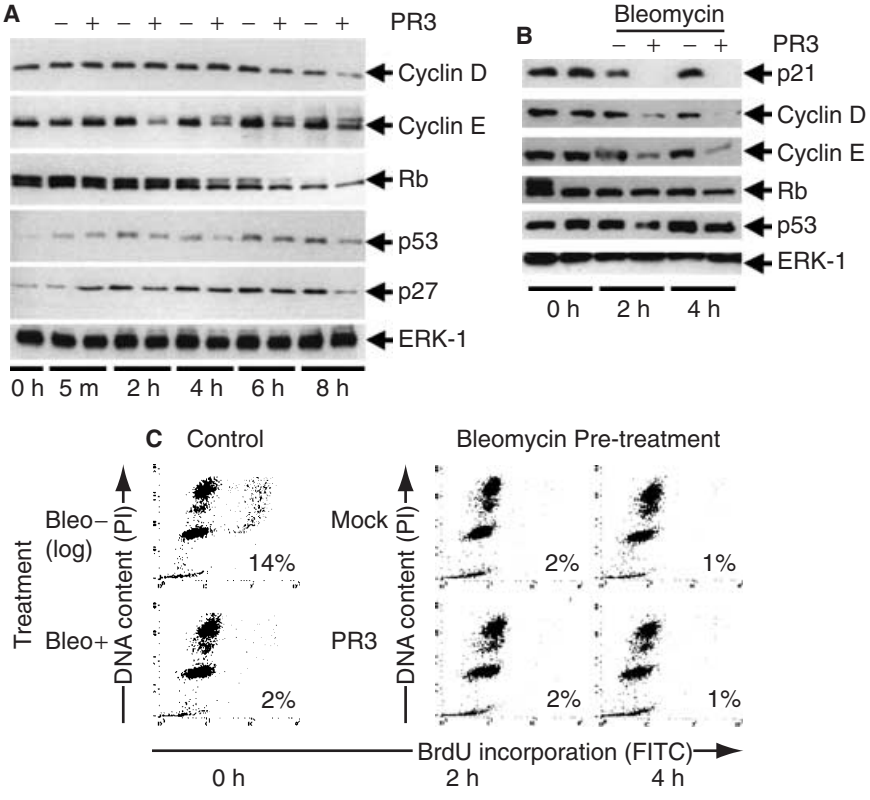

Fig. 3. Proteinase 3 (PR3)-treated endothelial cells reside primarily in G_0/G_1 stage of the cell cycle. (*A*) Immunoblot analysis of asynchronous cell populations of human umbilical vein endothelial cells (HUVEC) exposed to PR3, using antibodies specific for cell cycle–related proteins. (*B*) Enhanced PR3 effects on endothelial cells arrested in G_0/G_1. Immunoblot analysis of cell cycle control proteins in cells treated with PR3 after 24 hours of bleomycin pretreatment. Extracellular-related kinase-1 (ERK-1) shown for standard protein loading. (*C*) Documentation that bleomycin is effective in inducing growth arrest in HUVEC.

S phase [27]. In the PR3-treated cells, we found that cyclin D levels were slightly decreased by 6 hours, while cyclin E levels decreased by 2 hours. Because Rb is a substrate of cyclin D/Cdk2 and cyclin E/Cdk2 at the G_1/S transition, we examined Rb phosphorylation. An accumulation of hypophosphorylated Rb was more pronounced in PR3-treated cells by 4 hours, indicative of early G_1. p53 levels decreased in PR3-treated cells by 2 hours. Overall, p27 levels were relatively unchanged, and notably, no cleavage product was detected using antibodies to either the N- or C-terminus of the molecule. The activity of PR3 on p21, without interfering with p27 levels in vivo, suggests that PR3 regulates p21 functions, but appears not to disrupt p27 in these cells. Of note, changes in protein abundance at the 8-hour time point reflect cell death. Based on these data, the majority of PR3-treated cells accumulate in the early G_1 stage of the cell cycle (i.e., before cyclin E expression and Rb phosphorylation).

We noted from the data in Figure 1 that not all p21 molecules were cleaved after PR3 treatment. One explanation would be that these cells were asynchronous and only the cells in a particular stage of the cell cycle are susceptible. We hypothesized that PR3 effects would be exacerbated in cells forced to accumulate in the G_0/G_1 stage of the cell cycle. To test this, cells were exposed to a chemotherapeutic drug, bleomycin, which activates p21-induced cell cycle arrest, 24 hours prior to PR3 treatment. Western blot analysis indicated that p21 was essentially undetectable in growth-arrested cells after PR3 exposure (Fig. 4B), indicating that cleavage was enhanced as compared to asynchronous cells (see Fig. 1A). Enhanced reduction in cyclin D and E levels was observed after exposure. As expected, Rb status in bleomycin-treated cells was hypophosphorylated and unchanged despite PR3 exposure, indicative of G_0/G_1. Bleomycin treatment alone resulted in increased p53 also as expected, but was diminished after PR3 exposure. Why this occurred will need to be investigated. Flow cytometric analysis confirmed that cells were responsive to bleomycin and were indeed arrested in the cell cycle as demonstrated by lack of BrdU-positive S-phase cells (Fig. 3C). From this set of experiments, we can conclude that susceptibility to PR3-induced effects is enhanced in cells undergoing p21-induced cell cycle arrest with accumulation in the early G_1 phase of the cell cycle.

PR3 alters subcellular localization of p21

Because the C-terminus of p21 contains a nuclear localization sequence, and because the active p21 is bound to nuclear cyclin/CDKs, we examined the intracellular distribution of p21 after PR3 exposure. Two hours after PR3 exposure, immunocytochemistry showed that immunoreactivity for p21 disappears from the nucleus (Fig. 4D to F) as compared to untreated controls (Fig. 4A to C).

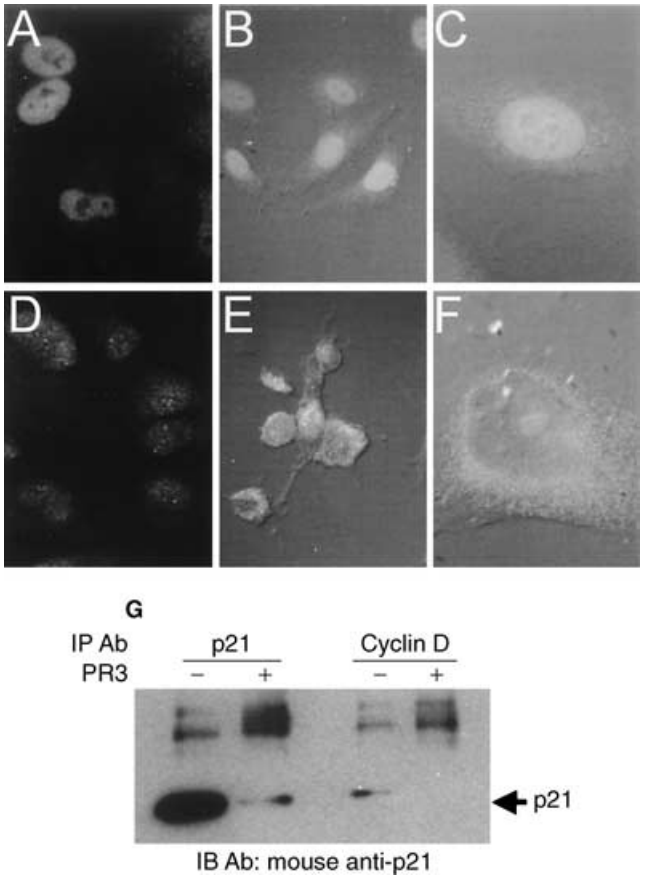

Fig. 4. Functional consequences of proteinase 3 (PR3)-cleaved p21 in human umbilical vein endothelial cells (HUVEC). Cells without (*A* to *C*) or with (*D* to *F*) PR3 for 2 hours were probed for p21. (A and D) 20×, (B and E) 40×, and (C and F) 100× magnification. (B, C, E, and F) Images shown with phase-contrast overlay. Immunofluorescence reveals loss of nuclear p21 in PR3-treated cells (representative of ~50% of cells). (*G*) Coimmunoprecipitations revealed that p21 is not complexed with cyclin D/cyclin-dependent kinase (CDK)2 in PR3-treated HUVEC. IP is immunoprecipitation; IB is immunoblot; and Ab is antibody.

Since p21 was not detected in the nucleus after PR3 exposure, we immunoprecipitated cyclin D, normally in the nucleus, in order to determine if p21 was in the complex. Full-length p21 was not observed complexed with cyclin D in the PR3-treated group (Fig. 4G). We can surmise that PR3 cleavage of p21 results in dissociation of p21 from the complex.

Analysis for commonality of substrates between PR3 and granzyme B

It is known that the T-cell serine protease granzyme B induces cyclin A/Cdc2 and cyclin A/Cdk2 kinase activity, and requires their induction in target cells for apoptosis to occur [28, 29]. Because PR3 cleaves p21 and has high structural similarities to granzyme B (Fig. 5A), we investigated whether granzyme B is capable of cleaving p21 in vitro. Sequence analysis of p21 fragments revealed a unique cleavage site on the molecule between amino acids Asp^{62} and Phe^{63} (Fig. 5B). A schematic representation of p21 presents the cleavage sites for granzyme B, PR3, and caspase-3 (Fig. 5C).

This work identified a substrate common to both PR3 and granzyme B. To further demonstrate this commonality, we asked if PR3 could cleave a known substrate of granzyme B, Bid [30]. Immunoblot analysis for Bid revealed that in fact PR3 cleaves Bid in vitro, although the fragment differed in size from that of granzyme B (Fig. 5D). These findings support our hypothesis that PR3 is a neutrophil homologue of T-cell granzyme B.

Does a p21 cleavage product exist in inflamed human tissue?

We hypothesized that p21 cleavage occurs during inflammation at sites of neutrophil infiltration. Free PR3 has been localized in inflamed tissue [2,3], and as a first attempt to address this hypothesis we asked if a PR3-like p21 cleavage product was present in inflamed tissue. Tissue samples from patients with inflammatory bowel disease provided a source to explore this question. Sections from a patient with Crohn's disease removed during colonic resection and anastomosis were homogenized and the total protein was examined for the presence of PR3. PR3 was detected and levels were highest in the inflamed section B (Fig. 6A). A distinct p21 fragment was detected using the p21-specific antibody (Fig. 6B). The size of the fragment is comparable to that generated in HUVEC (Fig. 6C). Interestingly, the tissue labeled section A was a small piece of bowel distal to the primary site of inflammation (section B). Based on appearance section A was assumed normal. However, the detection of a p21 fragment in this section could be an indication that some less obvious inflammation was occurring even at this distal site. Next, we examined inflamed proximal and transverse colon tissue obtained from a patient with ulcerative colitis during a colonectomy. We were successful in immunopurifying a p21 fragment of expected size from tissue homogenates, using a p21-specific antibody (Fig. 6D). This fragment was not detected in normal distal colon tissue.

It is known that caspase-3 cleaves p21 31 amino acids downstream of the PR3 cleavage site. Although the fragment appears to be generated by PR3 and not caspase-3, based on size, sequencing of the fragment is necessary and will need to be studied. Albeit, these data suggest that at sites of inflammation there is the potential for PR3 to cleave p21 in a manner similar to that observed in cell culture studies.

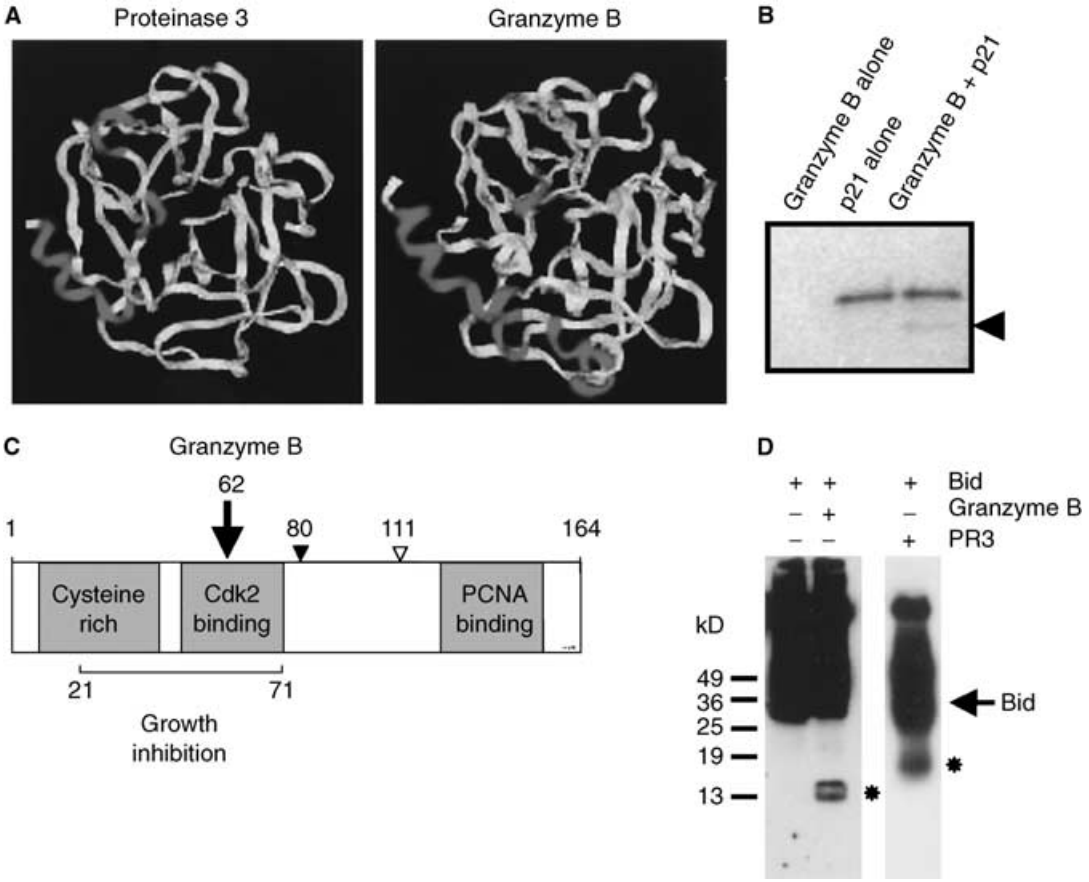

Fig. 5. A p21 cleavage product exists in inflamed human colonic tissue. Grossly normal (section A) and inflamed colon tissue (section B) were removed from a patient with Crohn's disease undergoing colonic resection and anastomosis. (*A*) Immunoblotting was performed for proteinase 3 (PR3). (*B*) Immunoblotting for p21 and truncated p21. (*C*) Human umbilical vein endothelial cells (HUVEC) with or without PR3 served as controls for the PR3-generated p21 fragment. (*D*) Inflamed proximal and transverse colon tissue and grossly normal distal colon tissue was removed from an ulcerative colitis patient undergoing colonectomy. p21 was immunoprecipitated from homogenized samples using either a non-specific antibody (−) or an anti-p21 antibody (+), and immunoblotted for p21.

DISCUSSION

A critical role for PR3 may be to augment the efficiency of caspase-dependent apoptotic events. We report here that PR3 cleaves p21 and Bid, two very important regulators of apoptosis, at signature sites. This function of PR3 appears to sidestep the requirement for activation of the caspase cascade (Fig. 7). Our results support a loss of function of p21 after PR3 cleavage based on the lack of p21 in the nucleus, and the lack of detectable p21 in complex with cyclin D [17]. We previously published that PR3 is present in the cytoplasm and the nucleus after endothelial cell entry [15] so it is likely that PR3 is cleaving both nuclear and cytoplasmic p21. Cleavage of p21 by caspases renders it inactive [17]. Even though PR3 cleaves p21 31 amino acids upstream of caspase-3, the critical domains of p21 are left intact suggesting that PR3-generated fragments would be functionally similar to those generated by caspase-3. We postulate that PR3 performs similar functions at sites of neutrophil and monocyte recruitment in tissue based on our findings that the p21 fragment is present in inflamed bowel tissue, coincident with detectable PR3.

It appears that the p21 molecule most susceptible to PR3 cleavage is the form that is actively functioning as a cell cycle inhibitor. In the presence of bleomycin, a cell cycle inhibiting drug that induces active p21, the p21 levels were ablated after PR3 treatment. It could be that changes in p21 status, to its active inhibitory role, facilitate cleavage. Interestingly, PR3 treatment resulted in reduced levels of cyclins D and E, indicative of the early G_0/G_1 stage of the cell cycle, which is biochemically similar to quiescence.

Although we detect roughly 15% to 20% apoptotic HUVEC after PR3 exposure, there are several explanations in which this percentage would be augmented. One would be that the receptor necessary for PR3 entry is only expressed on a subset of cells that is then upregulated during a stress response as in a focal inflammatory setting in vivo [31–35]. A second explanation could be that a cell cycle component is involved in PR3 effects.

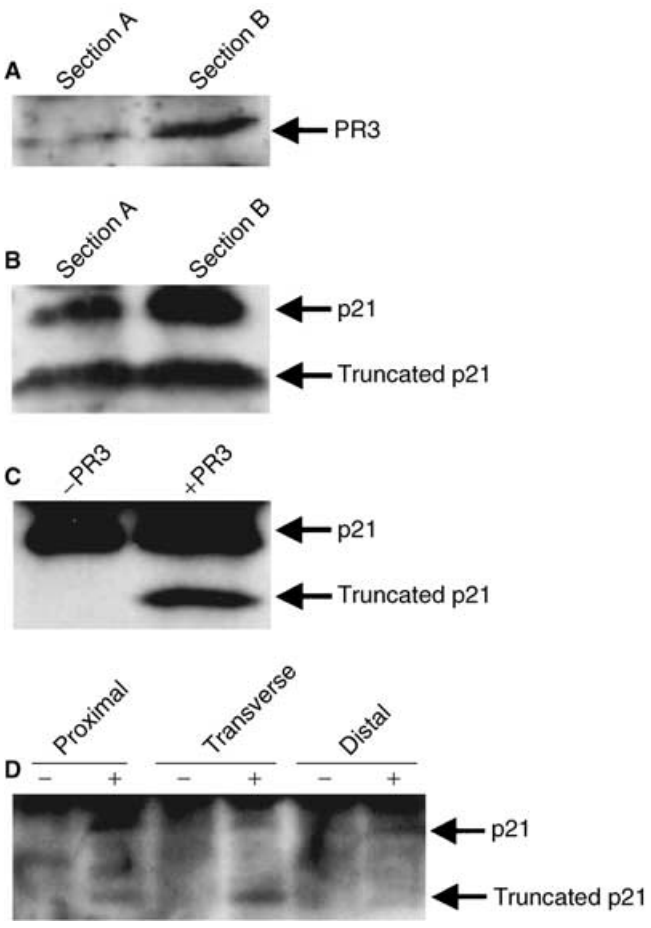

Fig. 6. Structurally similar proteinase 3 (PR3) and granzyme B cleave p21 and Bid. (*A*) Ribbon diagrams of PR3 and granzyme B with alpha-helical domains highlighted in red to illustrate structural similarity between these two proteases [55, 56]. (*B*) Recombinant p21 was incubated with granzyme B and resultant peptide fragments were visualized by Coomassie stain of an 18% sodium dodecyl sulfate (SDS) gel. Arrowhead indicates cleavage product that was excised for sequencing. (*C*) Schematic representation of human p21 depicts the cleavage sites of granzyme B (solid arrow), PR3 (closed arrowhead), and caspase-3 (open arrowhead). (*D*) Bid, immunopurified from human umbilical vein endothelial cells (HUVEC) lysate, was incubated with granzyme B or PR3. Both proteases cleaved Bid; however, cleavage products differed in size (∗).

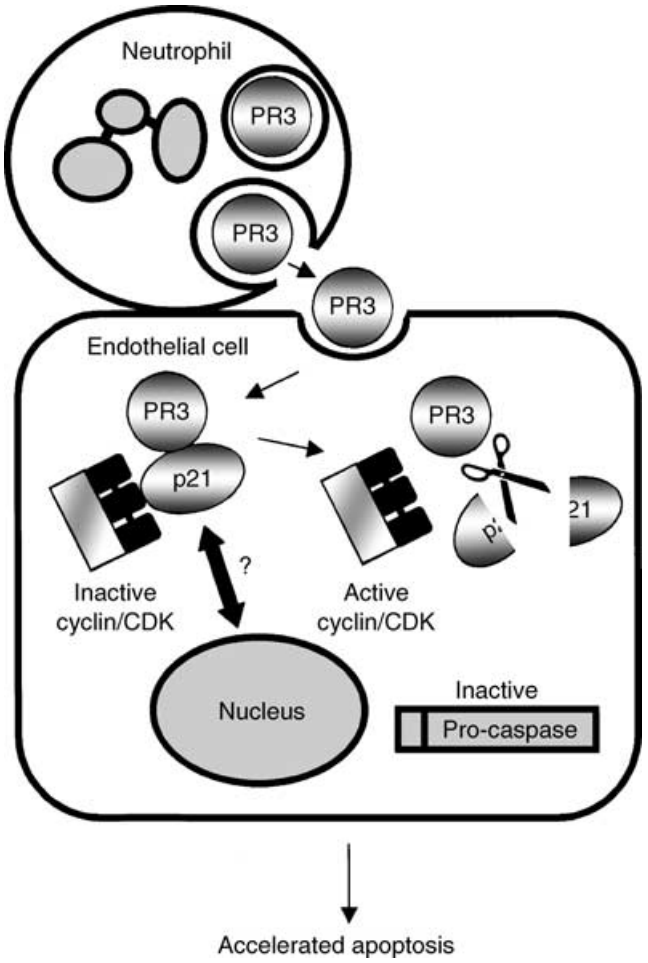

Fig. 7. Schematic representation of proteinase 3 (PR3)-mediated p21 inactivation. At sites of inflammation, stimulated neutrophils and/or monocytes undergo a respiratory burst releasing PR3, one component of the granule constituents. PR3 is internalized by endothelial cells, by an as yet undefined mechanism, and gains access to intracellular substrates. PR3 cleaves p21 in a region known to be susceptible to caspase 3 cleavage, although PR3 cleavage of p21 is caspase-independent. Cleavage results in loss of function of p21 with nuclear exclusion and activation of apoptosis. Because activation of the caspase cascade is not required for p21 cleavage, the kinetics of apoptotic activation would be accelerated. Subsequent phagocytosis of the apoptotic cell would aid in the resolution of inflammation, as both the injured endothelial cells and PR3 would be removed from the site.

Our studies indicate that PR3-mediated cleavage of p21 is more pronounced when cells are exposed to the cell cycle–perturbing chemotherapeutic drug, bleomycin.

PR3, also referred to as myeloblastin, expression appears to decrease during the course of normal myeloid differentiation [36]. Coordinately, p21 expression increases [37]. It appears that one role of PR3 is to cleave and presumably inactivate p21 during the differentiation process [38]. Furthermore, posttranscriptional down-regulation of PR3 mRNA inhibits proliferation and results in differentiation of myeloid progenitor cells into mature polymorphonuclear leukocytes [36, 39].

The question arises as to how PR3 enters endothelial cells and gains access to substrates in the cytoplasm of endothelial cells. One possibility is that PR3 could be taken up through receptor-independent mechanisms; however, current knowledge strongly implies that PR3 is internalized by a receptor-mediated mechanism [15, 16, 34]. In addition, Uehara et al [35] have reported that PR3 interacts with protease-activated receptor-2 on the surface of oral epithelial cells. Considering that electron micrographs of endothelial cells exposed to PR3 revealed existence of PR3 in the cytoplasm and in endocytic-like vesicles [15], we can hypothesize that it escapes the endocytic vesicle. This has been suggested by Witko-Sarsat et al [38] who recently described a role for PR3 in myeloid differentiation through PR3 cleavage of p21. They observed the

colocalization of PR3 and p21 in the cytosol in these cells. Their earlier findings that PR3 can exist in a plasma membrane-mobilizable secretory vesicle [40] led them to conclude that PR3 can escape from vesicles. Furthermore, PR3 is functional at both low pH and pH 7. PR3 exists in its native form as a functionally active holoenzyme within the neutrophil primary granule at a pH of 3 [41]. Baici et al [42] showed that PR3 is characterized by a slow gain of full activity with an increase of pH from 3.2 to 7.0. Since endothelial endosomes maintain an acidic pH [43], the data indicate that PR3 is enzymatically stable in this environment, and then becomes fully active once released by the endosome into the cytoplasm. In this case, PR3 could escape from the endosome once internalized just as other extracellular proteins are capable of doing [44].

We compared substrate specificities in vitro between PR3 and granzyme B, which is 37% homologous to PR3, for the following reasons. First, granzyme B is a highly characterized protease involved in cytotoxic T lymphocyte-mediated apoptosis. Second, granzyme B, like PR3, has been shown to be a caspase-like molecule in that it also cleaves caspase substrates. Finally, granzyme B-mediated apoptosis results in cyclin A-associated CDK activity, which is required for successful killing [28, 29]. This increased activity led us to postulate that p21 function could be altered by granzyme B, and in vitro, this is what was observed. Our observation that granzyme B cleaves p21 offers a mechanism for how this could occur. Our data and that of others demonstrate that truncation of p21 renders it inactive, releasing CDKs from inhibition by p21 [17, 38]. We suggest that PR3 is a neutrophil and monocyte homologue of the T-cell protease granzyme B, as supported by our findings that granzyme B cleaves p21 and that PR3 cleaves Bid [30].

PR3 and granzyme B may have evolved to combat viruses that have developed ways of delaying apoptosis to enable them to replicate and spread to uninfected cells. It is important to note that although neutrophils are generally associated with defense against bacterial invasion, they do play an important role in combating viral infection [45–48]. The granule secretory pathway is critical to immune surveillance against aberrant cells. Moreover, it is an intriguing concept that cleavage and/or inactivation of p21 by PR3 or granzyme B could possibly be a mechanism of immune-mediated removal of neoplastic cells. Recent clinical studies identified p21 expression as an indicator of poor survival in prostate cancer [49–52], and p21 has been reported to exert a protective effect against chemotherapeutic drugs in other types of cancer [20, 53, 54]. Based on our findings indicating that cells blocked in G_0/G_1 with bleomycin were more susceptible to PR3 effects, it may prove to be that tumor-directed PR3 treatment would enhance the efficacy of current treatment options.

Immune cells have evolutionarily acquired the capability to intervene into intracellular caspase cascades through highly specialized proteases, thus providing means to combat foreign microbes that override normal apoptotic signals. This specificity for caspase substrates constitutes an apoptotic effector mechanism of innate and adaptive immune cell proteases. Our studies have identified such a role for PR3 in normal diploid endothelial cells, and we are actively engaged in identifying mechanism(s) of cellular entry.

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